Effects of Ozone on Sciatic Nerve in Rat

Q. LIN, H. CHEN, C. LU, B. WANG, Y. ZHANG, X. HE, B. YU

Department of Orthopedics and Trauma, Nangfang Hospital, Southern Medical University; GuangZhou, Guangdong, China

Key words: ozone, sciatic nerve, safety

Summary

This study evaluated the influence of ozone on rat sciatic nerve structure and function. Thirty Wistar rats were randomly divided into six groups (n = 5). In groups I to IV, 1ml of ozone (O_3) 10 μg/ml, 30 μg/ml, 50 μg/ml, 8 0μg/ml was injected at the junction of gluteus maximus margin and lateral edge of the long head of biceps femoris respectively, in group V, 1 ml of pure O_2 was injected at the same point, and group V had puncture without any injection. Ozone was manufactured by an ozone generator (Ozone Line Co, Italy).

The rats were investigated by both gross measurement and behavioral changes. One day, one week and three weeks after injection, rat hindlimb footprints were measured and the sciatic nerve function index (SFI) was calculated, and after three weeks, all right sciatic nerves were exposed under anesthesia. Near neural stimulation of the rat sciatic nerve was calculated and nerve conduction velocity, latency and maximum amplitude recorded. Animals were sacrificed for pathology, and ipsilateral triceps surae were taken for wet weight. No serious behavioral abnormalities were observed in any animal. SFI comparison in the various times and various groups showed no significant differences ($p<0.05$), and nerve conduction velocity, latency and maximum amplitude difference amongst the groups was not significant ($p<0.05$). There were no abnormalities in peripheral nerves pathologically after injection. Our initial study suggests that ozone concentrations from 10 μg/ml to 80 μg/ml injected around rat's peripheral nerve will not cause serious sequelae or serious damage to the structure and function of peripheral nerve. This finding provides evidence of the safety of ozone injected around the peripheral nerve.

Introduction

In the treatment process of disc herniation, ozone could induce necrosis of the nucleus pulposus by its strong oxidation and it is still unknown whether the nerve root around ozone injection has any impact. The purpose of this study was to evaluate the safety of ozone injection by observing the effect of different concentrations of ozone on peripheral nerve structure and function, and to provide the clinical basis for experimental studies.

Materials and Methods

Materials

Examination animals: 30 Wistar rats in a SPF grade, weighing 180 g to 220 g, complex diet fed, were provided by the Examination Animal Center of Southern Medical University, (animal certification: 0030872). E80 ozonizer (OZONLINE, Italy), adjustable ozone level range of 1~80 μmol/ml, pore size 0.22 μm disposable gas filter needle (Department of Interventional Therapy, Nanfang Hospital); bottling oxygen (Nanfang Hospital); PcLab-UE processing system of biomedical signal.

Methods

Animal grouping: 30 Wistar rats were randomly divided into six groups: group I ozone 10, group II ozone 30, group III ozone 50, group IV ozone 80, group V oxygen group S control. In groups I to V 1 ml of ozone 10 μg/ml, 30 μg/ml, 50 μg/ml, 80 μg/ml oxygen was injected, and in group S, puncture at the same point without any injection.

Obtaining ozone in different concentrations:

bottling oxygen is connected with the E80 ozonizer that is turned on after connection by rubber tube. Then the E80 ozonizer is adjusted to harvest the expected ozone concentration and output. A syringe is fitted to the ozone outlet of the E80 ozonizer and pressed to obtain ozone which must not be taken by suction to avoid mixing with air.

Administration: After the experimental animals were anesthetized with continal of 3% by 30 mg/kg peritoneal injection, the operative field (right hind limb) was removed of hair, cleaned with wet absorbent gauze, disinfected with iodine and protected with normal drape, group I ozone 10, group II ozone 30, group III ozone 50, group IV ozone 80, group VI control. In groups I to IV 1 ml of ozone 10 μg/ml, 30 μg/ml, 50 μg/ml, 80 μg/ml was injected into the right ectogluteus respectively, and in group V puncture at the same point without any injection

Observation of appearance and praxiology of rats: action, feeding, claudication or not, infection in injection site or not, anabrosis or necrosis of toes or not, limitation of joints motion or not were observed.

SFI determination: according to three variables of Bain [1] measuring metapedes of rats: 1. podogram length, PL: the distance between heels and toes; 2. width between the first and fifth toes, TW; 3. inter-toes distance, IT: the distance between the second and fourth toes, values 1,2 and 3 were inserted in Bain's formula:

$$SFI = -38.3\left(\frac{EPL-NPL}{NPL}\right)+109\left(\frac{ETW-NTW}{NTW}\right)$$

$$+13.3\left(\frac{EIT-NIT}{NIT}\right)-8.8$$

to calculate SFI on scale ranging from 0 (normal) to –100 (complete disjunction of nerves).

Electrophysiology detection: after three weeks, under anesthesia the right sciatic nerve of rats was exposed to the use of PCLab-UE biomedical signal acquisition and processing system detected nearly sciatic nerve stimulation of the incubation period (LAT) and the maximum amplitude (AMP), then according to the formula to calculate the nerve conduction velocity (NCV).

Optical microscope observation and measurement of muscle wet weight: Excess of 3% sodium pentobarbital was used to kill the rats, after cutting about 2 cm of the sciatic nerve from the injection regions which prepared for fixing in formaldehyde solution and HE staining. Biopsy was then observed under the light microscope. Physical scales measured the wet weight of the cnemis whose triceps had been stripped.

Statistical analysis

The SPSS 13.0 packages were used to analyze the data. All the data obtained were expressed $\bar{x} \pm s$ by and analyzed by analysis of variance (ANOVA) and the significances were tested by t-test.

Results

Observation of appearance and praxiology of rats

One rat in group IV ozone 80 showed poor activity, slightly decreased appetite, no significant limp, injection of local non-infected area return to normal 1 day later. The remaining animals had no abnormal behavior. No rats in any group had toe ulcers or necrosis, and joint activities were unrestricted.

SFI comparison

The SFI values between groups showed no significant differences (P>0.05) at the same times. The SFI values of groups each time were not statistically significant (Figure 1, Table 1).

Neuroelectrophysiological examination and triceps surae muscle wet weight

The results of nerve conduction velocity (NCV) test of homogeneity of variance: *P*=.127, results analysed by one-way ANOVA F=2.684, P=0.046 showed a significant difference between groups. Results analysed by Student-Newman-Keuls for pairwise comparison of means of all groups were not statistically significant. Evoked potential latency (LAT) homogeneity of variance test P=0.028, results analysed by rank sum test, x^2=8.524, P=0.130, not statistically significant. Results of comparison of the largest amplitude (AMP) and triceps surae wet weight (WW) between the groups showed no significant differences (P>0.05) (see Table 2).

Observation of the general sample

There was no adhesion between the sciatic nerve and surrounding tissue and no obvious

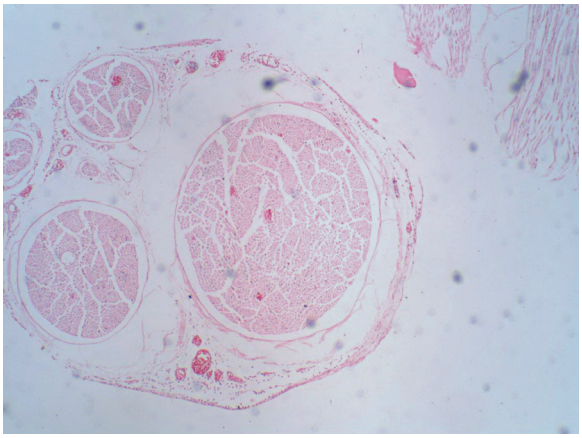

Figure 1 Sciatic nerve in each ozone group; HE staining (× 200): slightly fuzzy epineurium, perineurium smooth integrity of connective tissue in a small amount of cell infiltration, nerve fibers still neatly arranged with no obvious shrinkage, and no degeneration necrosis.

Table 1 **SFI group comparison at different times after ozone injection (mean + SD).**

Group	SFI value			Sum	F	P
	1 day	1 week	3 weeks			
Ozone 10	–10.72±4.85	–10.72±4.85	–10.72±4.85	–10.72±4.49	0.000	1.000
Ozone 30	–9.95±4.85	–9.09±4.54	–6.47±3.11	–8.51±3.74	1.211	0.332
Ozone 50	–13.71±4.14	–10.72±4.14	–10.72±4.63	–13.42±4.01	0.069	0.934
Ozone 80	–9.83±3.57	–5.58±4.85	–9.77±5.79	–8.39±4.35	1.172	0.219
Oxygen	–7.89±2.26	–9.14±3.45	–10.81±3.01	–9.28±3.00	1.235	0.325
Control	–11.38±2.71	–11.28±5.96	–12.64±5.69	–11.76±4.68	0.115	0.893
Sum	–10.58±3.71	–9.92±4.70	–10.54±4.74	–10.35±4.37	0.442*	0.589*
F	1.462	1.947	1.247	1.742*	(F=1.219, P=0.317)#	
P	0.239	0.124	0.319	0.167*		

** The main effect of* F *statistics and* P *values; # interactive effects and the* F *statistic* P *value.*

hyperemia, edema with white color, slightly green, flexibility. The neural surface was smooth.

Observation of the nerve situation under light microscope

Compared with the control group, each group of ozone epineurium was slightly fuzzy, but perineurium, nerve bundles and nerve fibers had no obvious injury, all ozone group performances were similar (Figure 1).

Discussion

Ozone, a kind of strong oxidant composed of three oxygen atoms, is non-persistent and its half-life period is about 20 min at normal temperature. It decomposes and dissolves in water easily. O_3 can restrain inflammatory cell factors, activate cycloxygenase, and reduce the stress response to histiocytic oxidation, increasing the histiocytic capability of resisting oxidation and free radicals. It can also scavenge the free radicals formed by chronic inflammation, serve as a painkiller and is anti-inflammatory [2]. However its role is twofold [3], high concentrations can cause oxidative damage, whereas too low a concentration will not achieve any therapeutic effect.

The present study on ozone focused on its effect on blood and blood cells, namely red blood cells. Although the buffer system in plasma, as well as a series of anti-oxidants, such as vitamin C, uric acid and glycoprotein, show the role of ozone in quickly reducing ozone activity, there is still a part of the ozone in the plasma that generates reactive oxygen species (ROS) which are neutralized by the plasma antioxidant system in 30 seconds to one minute. Blood experiments *in vitro* showed that ROS could trigger a number of biochemical pathways. When ozone comes into with plasma in plasma, it dissolves

Table 2 **NCV, AMP and WW comparison in each group after ozone injection (mean + SD).**

Group	NCV(m/s)	AMP(mV)	Triceps surae wet weight (g)
Ozone10	24.45±1.29	0.93±0.10	2.86±0.06
Ozone30	21.41±2.01	0.80±0.13	2.91±0.05
Ozone50	23.13±1.62	1.02±0.17	2.83±0.05
Ozone80	21.94±2.84	0.89±0.27	2.83±0.06
Ooxygen	24.77±0.69	0.81±0.23	2.86±0.08
Control	23.96±2.00	0.85±0.14	2.86±0.05
F	2.684	1.025	1.083
P	0.046*	0.425#	0.395#

** P<0.05, the differences between the groups were significant; # P>0.05, the differences between the groups were not significant.*

and quickly reacts with antioxidants and polyunsaturated fatty acids generating a series of compounds such as hydrogen peroxide and lipid peroxidation products one of which, 4-hydroxynonenal, activates the physiological function of blood, endothelial cells and real cells to regulate and treat various diseases [4]. Bocci [1,2] found that when the ozone concentration is less than 80 μg/ml, the buffer system and antioxidant system in blood and red blood cells can resist the damage to tissues and cell by ozone, thus fulfilling ozone treatment. With the increase in ozone concentration, more pronounced injury occurs.

There is also a certain amount of buffer substances and antioxidants in the subarachnoid space, joint cavity and abdominal cavity. When ozone is present in that space, the buffer substances and antioxidants can be rapidly activated to impose an antioxidant effect on organ damage. Tian et al. [5] reported that a 10 ml injection of 90 μg/ml ozone into pig subarachnoid space at 30 minutes, one week, one month and three months elicited no behavioural changes in biochemical markers in cerebrospinal fluid or significant structural change in the spinal cord under the naked eye and a microscope, thereby indicating that the buffer system and anti-oxidation system in cerebrospinal fluid could resist the injury to central nervous system by ozone. Zhang et al. [6] designed an experiment of intrathecal injection with three ozone concentrations in rabbits and found no impact on rabbit behaviour. They found SOD in cerebrospinal fluid was increased in the O_2 group and the ozone group compared with the control group. The concentration of MDA in cerebrospinal fluid was decreased in the O_2 group and O_3 group with low concentrations four hours after injection. MDA in cerebrospinal fluid in the O_3 group with high concentrations was increased one and a half hours after the injection. SOD/MDA was increased at all times after injection of O_2 and a low concentration of O_3, SOD/MDA was reduced ($P<0.05$) one and a half hours after the injection of high concentrations of ozone, suggesting that intrathecal injection of high O_3 concentrations in rabbits would have a potential toxic impact on the central nervous system.

Ozone has a wide range of applications in treatment for lumbar disc herniation, with a certain concentration of O_2-O_3 injected around the nerve root [7]. Controlled clinical studies have shown that a closed treatment using O_2 and O_3 to the nerve root was more effective than the use of non-steroidal hormone, and had no cumulative effects [8]. Due to the different structure of peripheral nerve compared with blood cells, spinal cord and other central nervous system structures, the peripheral nerve does not have the antioxidants in tissue fluid and tissue fluid to resist ozone oxidation, therefore the effect of different concentrations of ozone on peripheral nerve structure, function, is currently unknown, and no studies have been reported to date.

The present study explored the ozone effect on peripheral nerves by the design of different concentrations of ozone assessing the impact on rats' behaviour and the structure and function of their peripheral nerves to define the safety of ozone therapy on peripheral nerve-related diseases.

According to the study on ozone effects on red blood cells, when the ozone concentration was stronger than 50 μg/ml but less than 80 μg/ml, patients felt obvious pain in the treatment of partial ozone injection which increased with increases in gas concentration. So the concen-

tration of 20 μg/ml to 50 μg/ml is generally used in clinical practice. The clinical application of ozone would generally be divided into three groups: a low concentration of 10 μg/ml to 30 μg/ml, a mild concentration of 30 μg/ml to 50 μg/ml and a high concentration of 50 μg/ml to 80 μg/ml [4]. The concentrations applied in this experiment were 10 μg/ml, 30 μg/ml, 50 μg/ml and 80 μg/ml, commonly with a clinical ozone generator providing ozone E80.

This experiment suggested that ozone of all the different concentrations had no significant impact on rats' behaviour. The concentrations of 10 μg/ml, 30 μg/ml and 50 μg/ml also had no significant impact on the structure of the sciatic nerve. But the concentration of 80 μg/ml might have an impact on the epineurium, as surrounding connective tissue had a small amount of cell infiltration, but the perineurium and endometrial were integral, and nerve fibres arranged in order with no significant nerve fibre atrophy or degeneration. None of the ozone concentrations had a significant impact on the function of peripheral nerves, which may be due to the following: 1) the speed of ozone dispersion in the body is 20 times than that of oxygen, which makes sure the local concentration of ozone would not be too high after injection; 2) the sciatic nerve is composed of the endoneurium, perineurium and epineurium that is also covered by connective tissue. While the ozone is injected into peripheral nerves, it first reacts with peripheral tissue and epineurium, which largely reduces the oxidation ability of ozone. So it is unlikely that ozone would directly damage the nerve fibres due to the multiple layers of membrane structures. The concentration of 10 μg/ml to 80 μg/ml appeared safe in the treatment of peripheral nerve disease.

The experiment in this paper was conducted under the conditions of perineurium and epineurium being intact. Therefore, it is suggested that for a perineural injection of ozone, the patient should not be anaesthetized to block the peripheral nerve. The physician should slowly insert the needle and observe the patient's reaction to avoid the needle directly entering the nerves and causing mechanical injury to the nerve and oxidative damage to nerve fibres.

References

1 Bain JR, Mackinnon SE, Hunter DA. Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat. Plast Reconstr Surg. 1989; 83: 129-138.
2 Bocci V. Ozone-A New Medical Drug. Dordrecht: Springer; 2005.
3 Bocci V. Ozone as Janus: this controversial gas can be either toxic or medically useful. Med Inflamm. 2004; 13 (1): 3-11.
4 Dianzani MU. 4-hydroxynonenal from pathology to physiology. Mol Aspects Med. 2003; 24: 263-272.
5 Tian JL, Zhang JS, Xiao YY, et al. Changes of CSF and spinal pathomorphology after high-concentration ozone injection into the subarachnoid space: an experimental study in pigs. Am J Neuroradiol. 2007; 28: 1051-1055.
6 Zhang W, Fu Z, Xie JT, et al. Intrathecal injection of ozone on rabbit behavior and cerebrospinal fluid superoxide dismutase and malondialdehyde levels. Chin J Anesthesia. 2006; 06: 552-554.
7 Andreula CF, Simonetti L, Santis F, et al. Minimally invasive oxygen-ozone therapy for lumbar disk herniation. Am J Neuroradiol. 2003; 24: 996-1000.
8 Bonetti M, Fontana A, Cotticelli B, et al. Intraforaminal O_2-O_3 versus periradicular steroidal infiltrations in lower back pain: randomized controlled study. Am J Neuroradiol. 2005; 26: 996-1000.

Bin Yu, MD, PhD
Department of Orthopedics & Trauma
Nanfang Hospital, Southern Medical University
No. 1838 North Guangzhou Avenue
Guangzhou; 510515 China
E-mail: yubinol@163.com

Xiaofeng He, MD, PhD
Department Interventional Department
Nanfang Hospital, Southern Medical University
Tel.: +86 20 62787500
Fax: +86 20 62787500
E-mail: ozonetherapy@126.com